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Note

Rapid and sensitive FAME analysis of bacteria by cold trap injection gas chromatography

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Abstract

Whole cell fatty acid analysis is commonly used to identify bacteria. A cold trap, using a commercially available device that directs a stream of compressed air across a section of the GC column, is used to focus peaks at the head of the column. When combined with a rapid sample processing method that uses smaller volumes of solvents, it becomes possible to correctly identify bacteria from 1 to 2 mg of biomass.
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The Microbial Identification System (MIS, MIDI, Newark, DE) for fatty acid methyl ester (FAME) analysis is a standard method for identification of microorganisms. Whole cell fatty acids are converted to methyl esters and analyzed by gas chromatography. The fatty acid composition of the unknown is compared to a library of known organisms in order to find the closest match. Originally, the system required 40 mg of biomass (wet weight) and had chromatographic run times of 20.8 min (MIDI, 1992). Sensitivity was limited by the final sample volume (1.25 ml) and the chromatographic split ratio (100:1).

We introduced a splitless method with much higher sensitivity but somewhat longer chromatographic run time that was useful for analysis of large single colonies (Buyer, 2002a). We then developed a fast method using a smaller chromatography column with a run time of 6.65 min and a method for rapid sample processing (Buyer, 2002b). This method required a split ratio of 200:1, reducing sensitivity, but this was partly compensated for by the much smaller final sample volume (300 μl) and the sharper peaks. In subsequent research, we were able to reduce the run time down to 3.3 min (Buyer, 2003). MIDI introduced methods which used the same size column as their original methods but faster temperature gradients and higher gas flow rate. These rapid methods (RTSB50, RCLN50, RBTR50) had run times of 5.83 min and, with split ratios of 40:1, required 20 mg of biomass (MIDI, 2002).

In this paper, we show that cold trapping at the head of the chromatography column, using a device that focuses peaks by cooling a section of the column with a stream of compressed air, can be used to lower the split ratio of the MIS rapid methods to 10:1 while only increasing chromatographic run time by 0.1 min. When combined with the smaller final sample volume of the rapid sample processing method, it becomes possible to correctly identify bacteria while using only 1–2 mg of biomass.

Standard strains of bacteria, reagents and sample preparation techniques were previously described (Buyer, 2002b). Methanol and hexane were capillary

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Table 1
Gas chromatographic conditions for RTSB50 and FAST6 methods

Item	RTSB50 method	FAST6 method
Column	Ultra 2, 25 m long × 0.2 mm ID × 0.33 μm film thickness	Ultra 2, 25 m long × 0.2 mm ID × 0.33 μm film thickness
Carrier gas	Hydrogen, 1.3 ml/min, constant flow	Hydrogen, 1.3 ml/min, constant flow
Oven program (°C)	170 to 288 at 28/min 288 to 310 at 40/min Hold 1.25 min	170 hold 0.1 min 170 to 288 at 28/min 288 to 310 at 40/min Hold 1.25 min
Split ratio	40:1	10:1
Injection volume	2 μl	2 μl
Valve 5 (AirSharp)	None	Initially on Off 0.1 min On 5.8 min
Run time	5.83 min	5.93 min

GC grade while methyl *tert*-butyl ether was pesticide residue analysis grade (Aldrich, Milwaukee, WI). Deionized water was extracted with reagent 3 (1:1 hexane/methyl *tert*-butyl ether) and stored under reagent 3. Glassware was baked at 500 °C overnight in a muffle furnace. The chromatographic system consisted of an Agilent 6890 Plus gas chromatograph (Agilent Technologies, Palo Alto, CA) with a 7683 autoinjector, split–splitless inlet, flame ionization detector and electronic pressure control. The inlet was equipped with a Merlin microseal septum (Merlin Instrument, Half Moon Bay, CA). The system was controlled with Chemstation (Agilent) and Sherlock (MIDI) software.

Cold trapping was accomplished with an AirSharp trap (SGE, Austin, TX). The AirSharp blows ambient temperature air across a short section of the chromatography column inside the column oven, thus lowering the temperature of that section of the column and trapping any peaks in that section. When the air flow is turned off, that section of the column heats up rapidly and the peaks elute. A solenoid valve, under software control, is used to turn the air on and off. The AirSharp was installed near the head of the column, as close to the injector as possible, to trap FAMEs after injection.

The method presented here, FAST6, was based on the MIDI method RTSB50 (Table 1). The AirSharp air flow was turned on initially, turned off at 0.1 min and turned back on at 5.8 min. The oven temperature program was modified by adding a 0.1-min hold after injection in order to allow time for trapping to occur. The injection split ratio was changed from 40:1 to 10:1. Total chromatographic run time was 5.93 min.

Calibration runs are presented in Fig. 1 for the parent method, RTSB50, and the new method, FAST6. In FAST6, the solvent peak was much broader than in

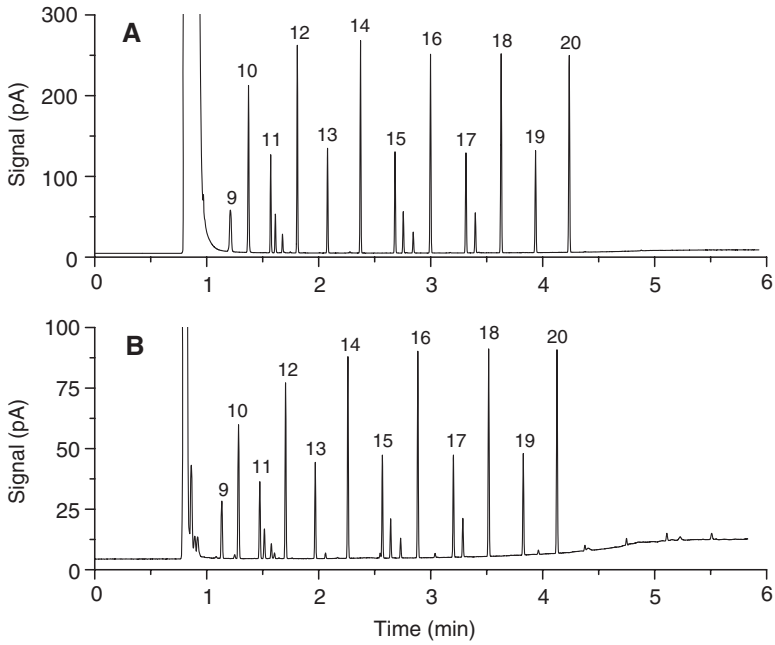

Fig. 1. Chromatograms of the MIDI calibration mix run by the (A) FAST6 method and (B) RTSB50 method. In (A), MIS Calibration Mix 1 is diluted 1:10, while in (B) MIS Rapid Calibration Mix is used without dilution. Straight-chain fatty acids are numbered, while hydroxy fatty acids present in the calibration mix are not labeled.

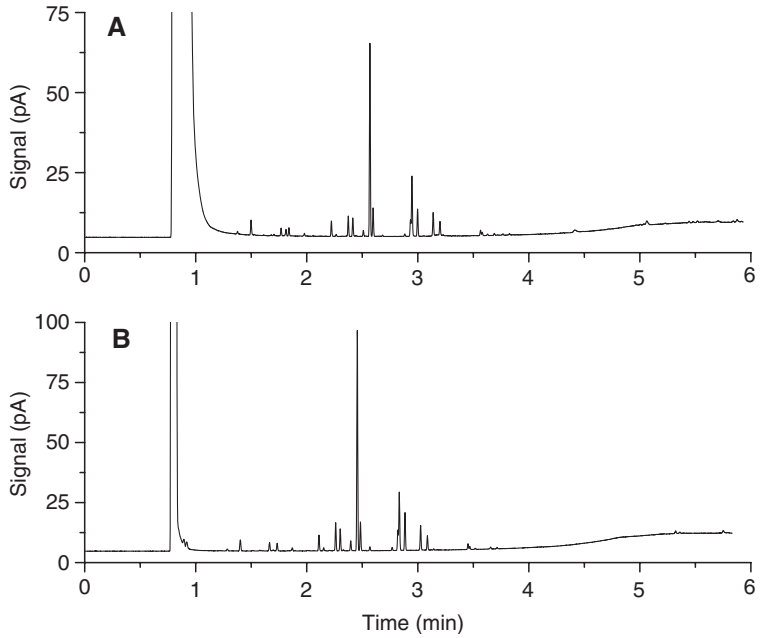

Fig. 2. Chromatograms of fatty acid methyl esters from *S. maltophilia* run by (A) FAST6 and (B) RTSB50.

RTSB50 and the first calibration mix peak, 9:0, was slightly broader. With a smaller split ratio, such as 8:1 or 9:1, the solvent and 9:0 peaks began to merge, so 10:1 was the lowest split ratio we found to be useful. The system calibrated successfully with FAST6 without making any changes to the peak naming table. However, because of the additional sensitivity, the calibration mix had to be diluted before using with FAST6. We used MIS Calibration Mix 1 (MIDI) diluted 1:10 with reagent 3.

Samples were prepared by the standard MIS method, using approximately 20 mg of biomass (wet weight), for use with the RTSB50 chromatographic method. This method uses screw-cap test tubes and reagent volumes of 1–3 ml. Samples for the FAST6 chromatographic method were prepared with the rapid sample processing method (Buyer, 2002b) using approximately 1–2 mg. This method uses 1-ml microtubes arrayed identically to a 96-well microplate, sample volumes of 0.15–0.60 ml and multichannel pipetters. Chromatograms for *Stenotrophomonas maltophilia* are shown in Fig. 2. The correspondence between the two chromatograms is quite clear. An additional peak in the FAST6 run, identified by the software as 12:0, is visible at about 1.8 min in Fig. 2A and indicates contamination (see below). *S. maltophilia*

Table 2
Identification of bacteria using the RTSB50 and FAST6 methods

Organism	TSBA		Blood agar	
	RTSB50	FAST6	RTSB50	FAST6
Acinetobacter baumanii	0.802[a] (14.5%)[b]	0.790 (9.3%)	0.693 (18.2%)	0.800 (16.2%)
Bacillus cereus	0.857 (12.3%)	0.508 (16.7%)	0.740 (18.5%)	0.714 (14.3%)
Chryseobacterium balustinum	0.907 (0.9%)	0.859 (6.5%)	N	N
Pseudomonas aeruginosa	0.896 (9.4%)	0.822 (9.4%)	0.868 (8.7%)	0.861 (13.2%)
Pseudomonas fluorescens	0.811 (8.9%)	0.740 (7.5%)	N	N
Shewanella putrefaciens	0.682 (8.7%)	0.802 (8.8%)	0.732 (7.2%)	0.757 (10.8%)
Staphylococcus aureus	0.650 (14.7%)	0.633 (7.8%)	0.741 (9.0%)	0.713 (5.2%)
Stenotrophomonas maltophilia	0.916 (0.6%)	0.930 (2.4%)	0.763 (8.4%)	0.730 (10.3%)

Cultures grown on TSBA at 28 °C were compared to the RTSB50 library, while cultures incubated on blood agar at 35 °C were compared to the RCLN50 library. Four to six replicates were performed.

N: not tested.

[a] Library matches are expressed on a scale of 0 to 1, with a match of 0.5 or greater considered good to the species level.

[b] Percent standard deviation is parenthesized.

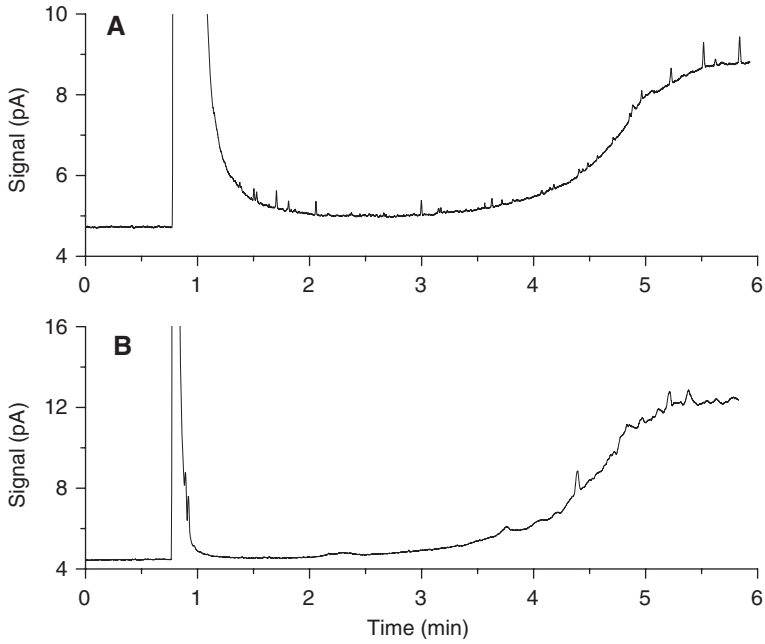

Fig. 3. Chromatograms of blanks. (A) FAST6, (B) RTSB50.

and other bacteria were identified similarly by FAST6 and RTSB50 (Table 2). FAST6 was useful for bacteria grown on tryptic soy broth agar at 28 °C and on blood agar at 35 °C (Table 2).

Blank runs are presented in Fig. 3. Several small peaks can be seen in the FAST6 blank which are not observed in the RTSB50 blank. The FAST6 blank runs typically had named areas of 1000 to 3000, and the fatty acids 12:0 and 16:0 usually accounted for most of the area. Blanks of this quality did not interfere with correct identification of bacteria. However, as sample size was lowered further, the percentage of named peak area contributed by the contaminants became larger and the quality of the library match declined.

Sensitivity was increased four-fold by reducing the split ratio from 40:1 to 10:1. Another approximately four-fold increase was obtained by using the rapid sample processing method, which reduced the final volume of the sample from 1.25 ml to 300 µl. Taken together these changes allowed us to reduce sample size from 20 mg to 1–2 mg or a 10–20-fold increase in sensitivity. The chromatographic method presented here is only 0.1 min longer than the MIS method it is derived from but far more sensitive. This method is not quite as sensitive as the splitless method developed earlier (Buyer, 2002a) but it is much faster. When compared to the rapid methods we previously published (Buyer, 2002b, 2003), this new method is much more sensitive and uses the standard MIDI column and injection liner but is slightly slower. Continued improvements in speed and sensitivity may make fatty acid analysis more useful in situations where sample size is limiting and rapid analysis is critical, such as bioterrorism agent detection.

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